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Supplementary Materials for

R-loops trigger the release of cytoplasmic ssDNAs leading to chronic inflammation upon DNA damage

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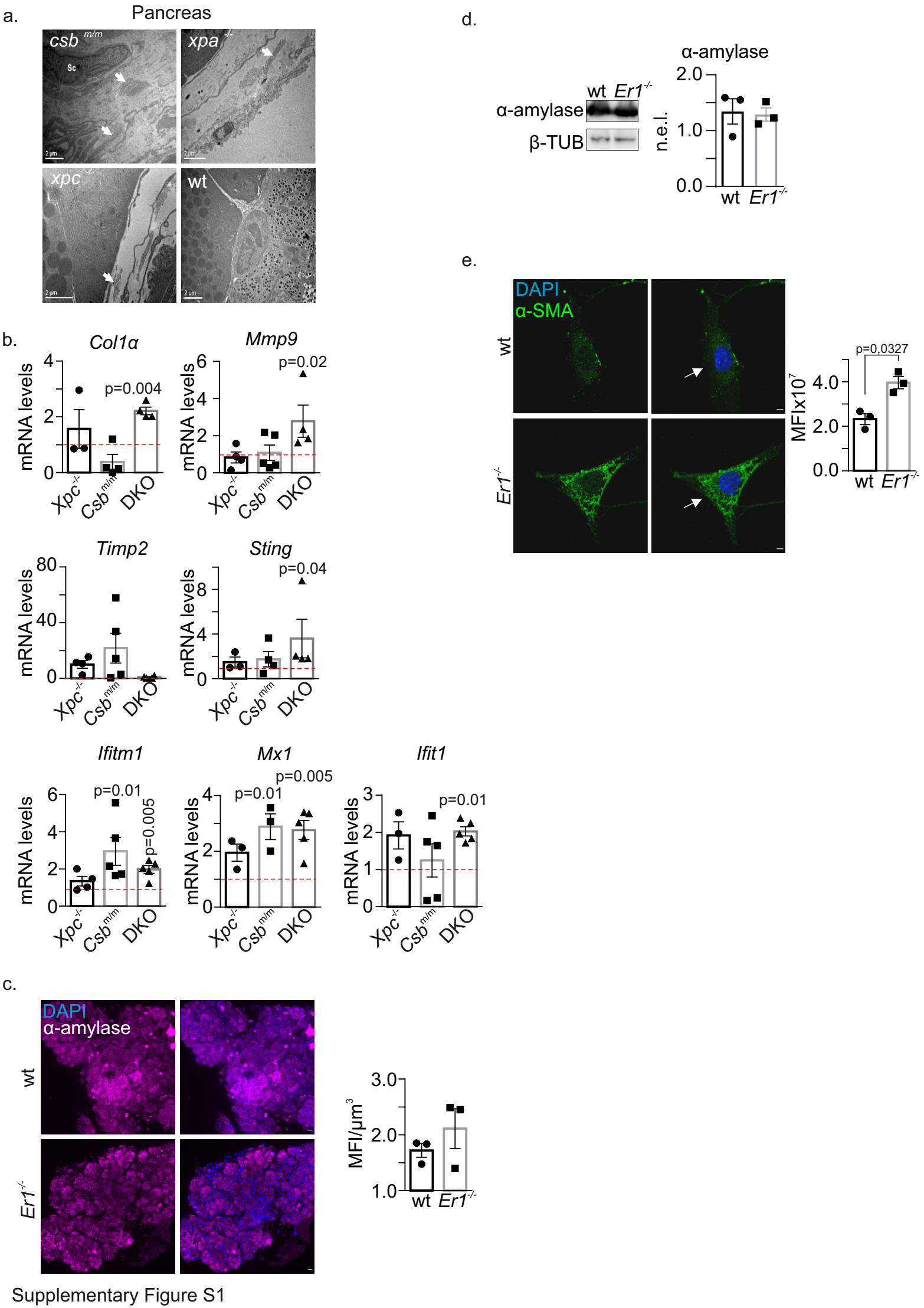
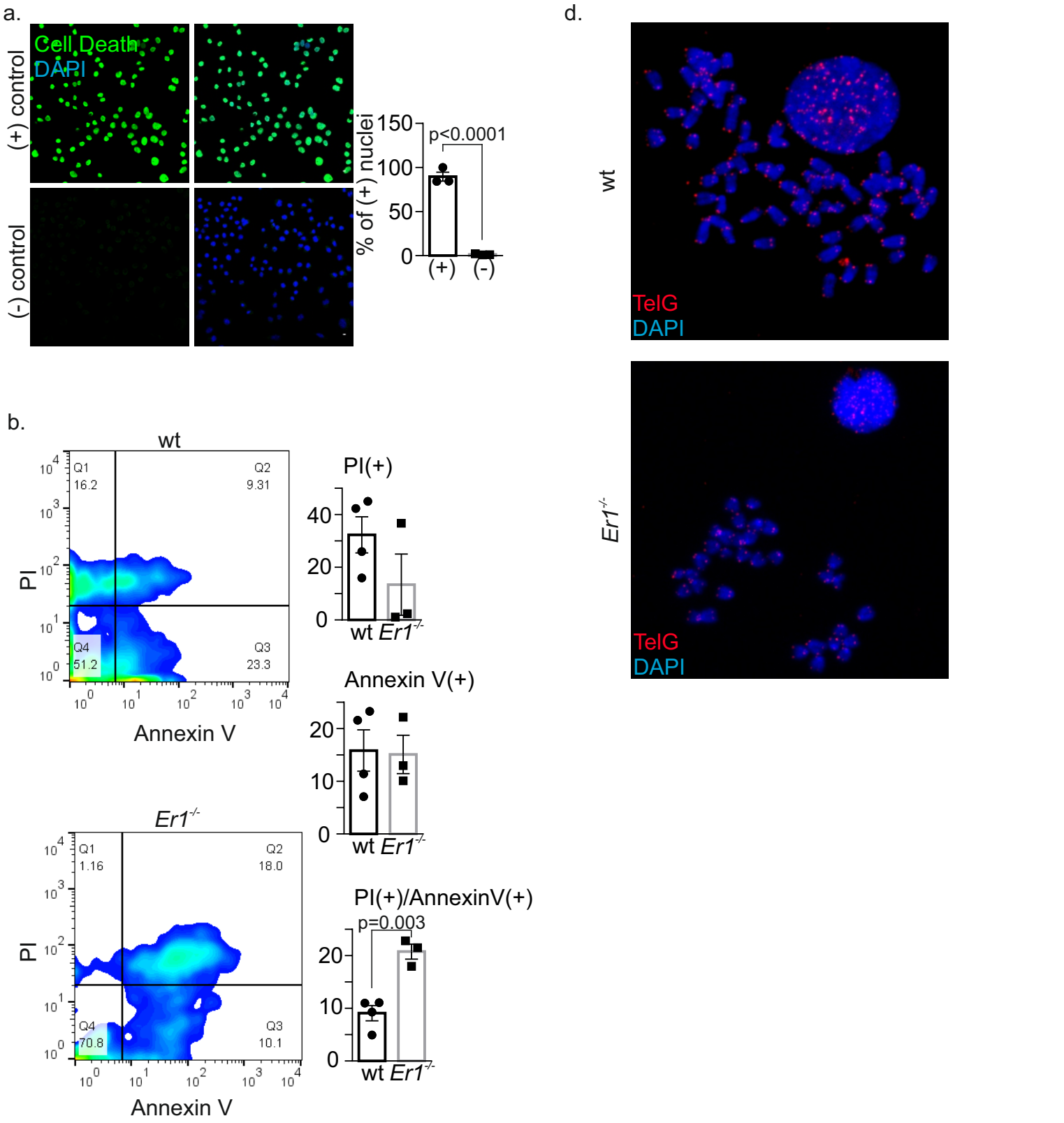
Other Supplementary Material for this manuscript includes the following:



Supplementary Figure S1

Fig. S1.

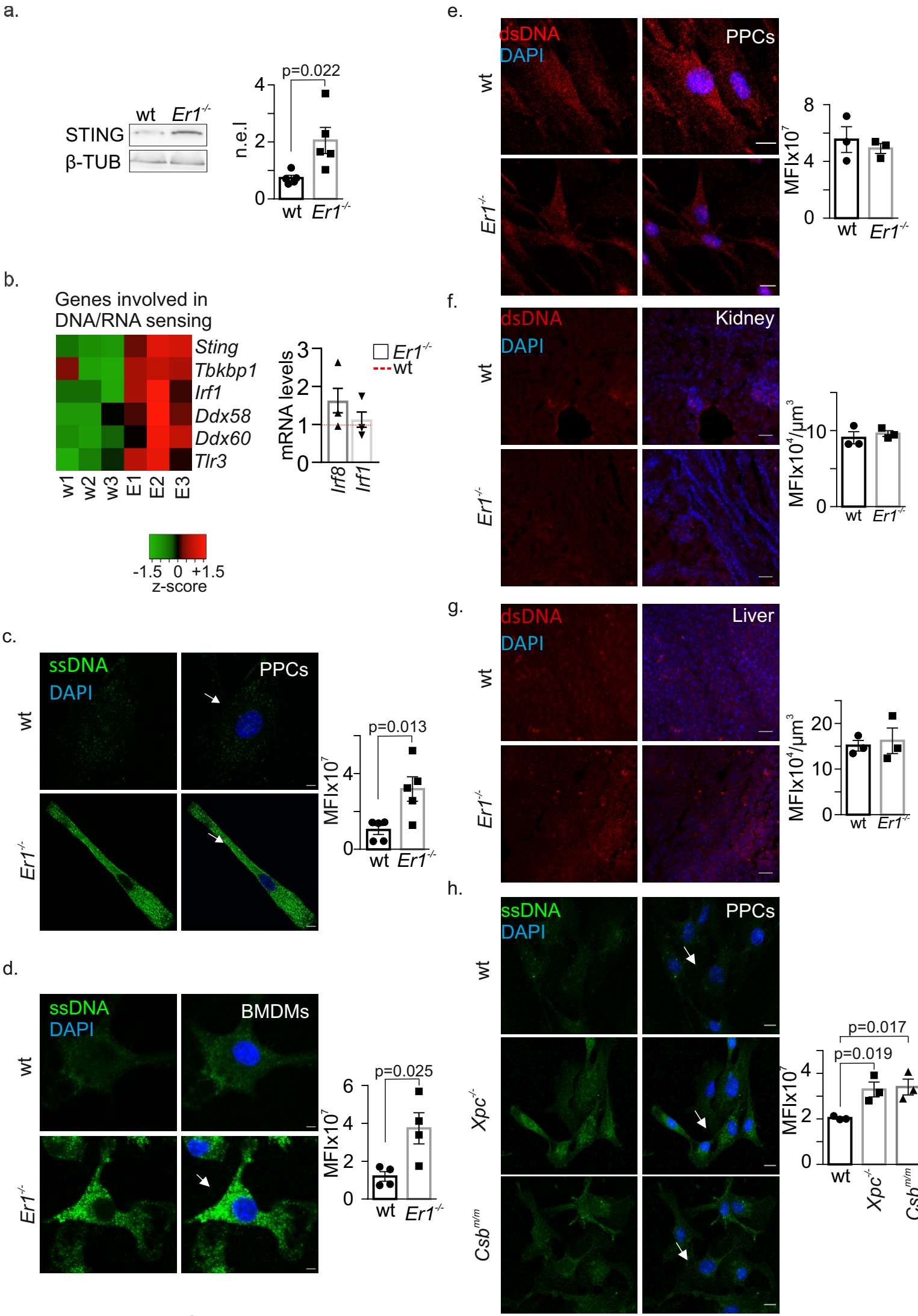
Supplementary Fig. 1. (A). Representative transmission electron micrographs (TEM) of *Csb*$^{m/m}$, *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ and wt pancreata. Arrowheads depict no difference in the collagen fibril network. **(B)**. *Col1a*, *Mmp9*, *Timp2*, *Sting*, *Ifitm1*, *Mx1* and *Ifit1* mRNA levels in *Xpc*$^{-/-}$, *Csb*$^{m/m}$, *Xpc*$^{-/-}$;*Csb*$^{m/m}$ (DKO) and wt pancreata. The red dashed line depicts the wt levels. **(C)**. Immunofluorescence detection of α-AMYLASE (purple) in wt and *Er1*$^{-/-}$ pancreatic tissue. The graph represents the mean fluorescence intensity (MFI) per μm^3 of tissue (n=3). **(D)**. Western blotting of α-amylase protein in whole-cell extracts. β-TUBULIN (β-TUB) was used as loading control (as indicated, n=3). The graph represents the β-TUB-normalized expression levels (n.e.l.) of α-AMYLASE protein in *Er1*$^{-/-}$ compared to wt controls. **(E)**. Immunofluorescence detection of alpha smooth muscle actin (α-SMA) in wt and *Er1*$^{-/-}$ PPCs (n=3). The graph represents the mean fluorescence intensity (MFI) per cell. Unless otherwise stated, all tissues and cells used are derived from P15 mice. White line is set at 5μm scale, unless otherwise indicated. Error bars indicate S.E.M. among n≥3 replicates. The significance is set at p-value ≤0.05 and where appropriate the exact p-value is shown (two-tailed Student's t-test).



Supplementary Figure S2

Fig. S2.

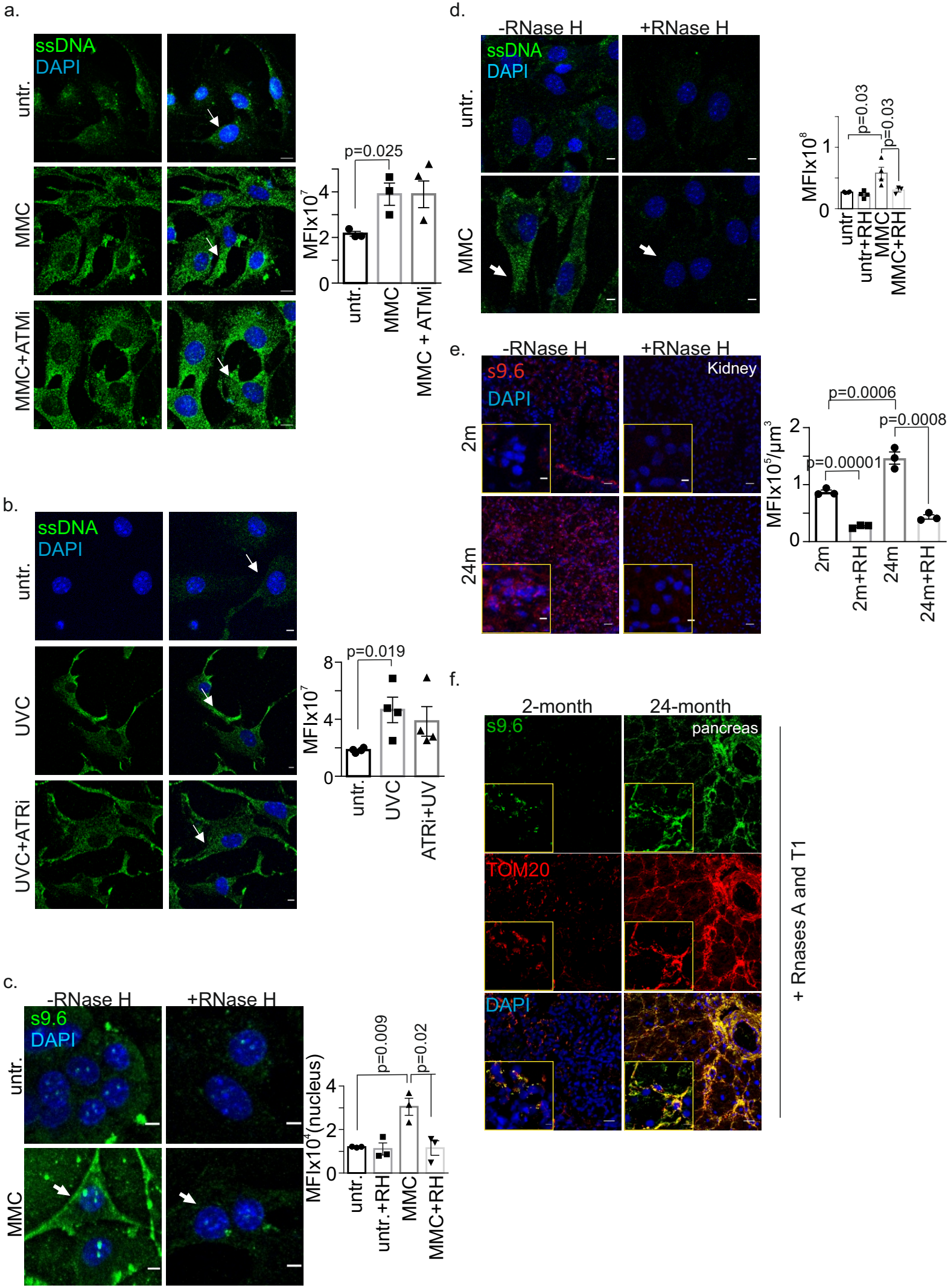
Supplementary Fig. 2. (A). Immunofluorescence detection of TUNEL-positive cells in untreated (- control) or DNase-treated PPCs (+ control) (n=3). The graphs depict the percentage of positive-stained cells in untreated (- control) or DNase-treated PPCs (+ control, lower). **(B)**. Fluorescence cytometry representative plots for Annexin V/Propidium Iodide (PI) in wt and *Er1*$^{-/-}$ pancreatic tissues. Graphs depict the percentage of PI+, AnnexinV+ and PI+/AnnexinV+ wt and *Er1*$^{-/-}$ pancreatic cells (n=3). **(C)**. *Tert* mRNA levels in wt and *Er1*$^{-/-}$ pancreata (n=3). The red dashed line represents the wt levels. **(D)**. Metaphase spreads from *Ercc1*$^{-/-}$ and wt PPCs. Chromosomes were stained with DAPI (blue) and telomeric DNA (red) was detected by FISH with a Cy3-conjugated PNA probe (n=3, metaphases >20/genotype). Unless otherwise stated, all tissues and cells used are derived from P15 mice. Error bars indicate S.E.M. among n≥3 replicates. The significance is set at p-value ≤0.05 and where appropriate the exact p-value is shown (two-tailed Student's t-test).



Supplementary Figure S3

Fig. S3.

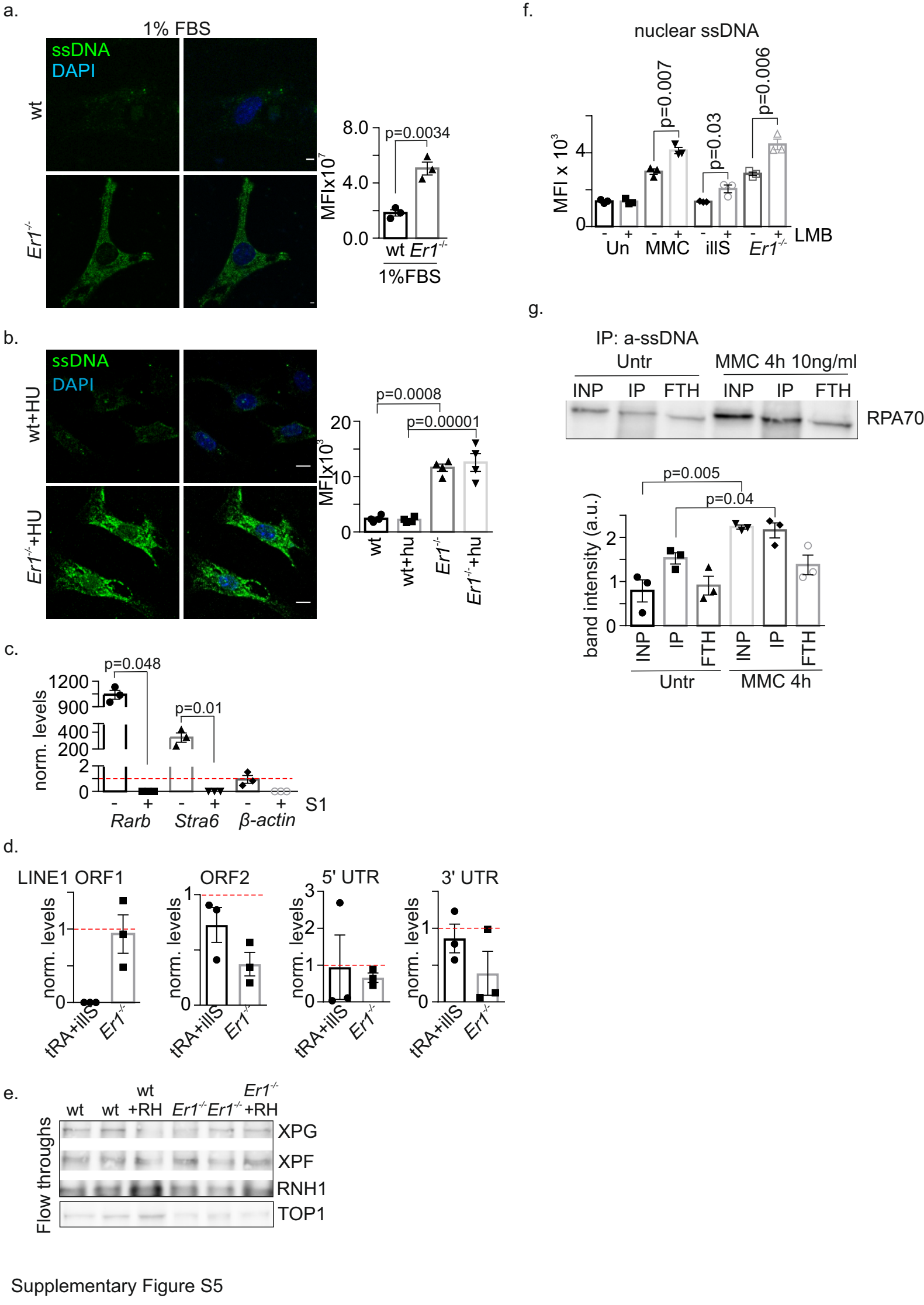
Supplementary Fig. 3. (A). Western blotting of STING protein in whole-cell extracts of wt and *Er1*$^{-/-}$ pancreata. β-TUBULIN was used as loading control (β-TUB, as indicated). The graph represents the β-TUB-normalized expression levels (n.e.l.) of STING protein in *Er1*$^{-/-}$ compared to corresponding wt controls (n=3). **(B)**. Heatmap of representative genes, upregulated in *Ercc1*$^{-/-}$ (E1-3) compared to wt (w1-3) pancreata, associated with DNA sensing processes (as indicated). The graph depicts *Irf8* and *Irf1* mRNA levels in wt and *Er1*$^{-/-}$ pancreata. The red dashed line represents the wt levels. **(C)**. Immunofluorescence detection of ssDNA in wt and *Er1*$^{-/-}$ PPCs (as indicated by arrows, n=5). The graph depicts the mean fluorescence intensity (MFI) per cell. **(D)**. Immunofluorescence detection of ssDNA in wt and *Er1*$^{-/-}$ BMDMs. The graph represents the mean fluorescence intensity (MFI) per cell (n=4). **(E)**. Immunofluorescence detection of dsDNA in wt and *Er1*$^{-/-}$ PPCs. The graph depicts the mean fluorescence intensity (MFI) per cell (n=3). **(F)**. Immunofluorescence detection of dsDNA in wt and *Er1*$^{-/-}$ kidneys. The graph depicts the mean fluorescence intensity (MFI) per cell (n=3). White line is set at 50μm scale. **(G)**. Immunofluorescence detection of dsDNA in wt and *Er1*$^{-/-}$ livers. The graph depicts the mean fluorescence intensity (MFI) per cell (n=3). White line is set at 50μm scale. **(H)**. Immunofluorescence detection of ssDNA in wt, *Xpc*$^{-/-}$ and *Csb*$^{m/m}$ pancreata. The graph depicts the mean fluorescence intensity (MFI) per cell (n=3). Unless otherwise stated, all tissues and cells used are derived from P15 mice. White line is set at 5μm scale, unless otherwise indicated. Error bars indicate S.E.M. among n≥3 replicates. The significance is set at p-value ≤0.05 and where appropriate the exact p-value is shown (two-tailed Student's t-test).



Supplementary Figure S4

Fig. S4

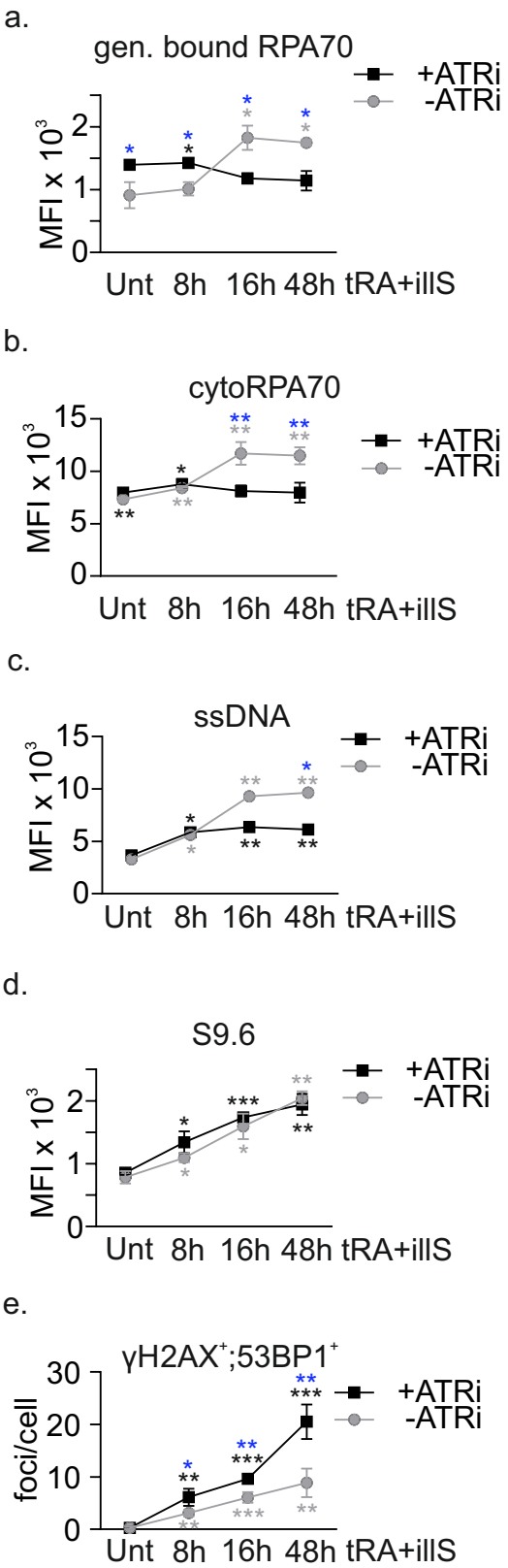
Supplementary Fig. 4. (A). Immunofluorescence detection of ssDNA in untreated, MMC-treated (10μg/ml, 4h) and MMC-treated wt PPCs, pre-cultured for 1h in the presence of 10μM ATM inhibitor (ATMi). The graph depicts the mean fluorescence intensity (MFI) per cell (n=3). **(B)**. Immunofluorescence detection of ssDNA in untreated, UV-irradiated (15J/m^2) and UV-irradiated wt PPCs, pre-cultured for 1h in the presence of 10μM ATR inhibitor (ATRi). The graph depicts the mean fluorescence intensity (MFI) per cell (n=4). **(C)**. Immunofluorescence detection of R-loops, by means of S9.6 antibody staining, in untreated and MMC-treated (10μg/ml, 4h) wt PPCs in the absence or presence of transfected recombinant RNase H. The graph depicts the mean fluorescence intensity (MFI) per cell nucleus (n=3). **(D)**. Immunofluorescence detection of ssDNA in untreated and MMC-treated (10μg/ml, 4h) wt PPCs in the absence or presence of transfected recombinant *E. coli* RNase H. The graph depicts the mean fluorescence intensity (MFI) per cell (n=3). **(E)**. Immunofluorescence detection of R-loops, by means of S9.6 antibody staining, in 2- and 24-months naturally aged kidneys in the absence or presence of RNase H. The graph depicts the mean fluorescence intensity (MFI) per cell nucleus (n=3). **(F)**. Immunofluorescence detection of R-loops, by means of S9.6 antibody staining (green) and mitochondria (a-TOM20 antibody, red) in 2- and 24-months naturally aged pancreata from wt mice treated with RNases A and T1 (n=3). Unless otherwise stated, all tissues and cells used are derived from P15 mice. White line is set at 5μm scale. Error bars indicate S.E.M. among n≥3 replicates. The significance is set at p-value ≤0.05 and where appropriate the exact p-value is shown (two-tailed Student's t-test).

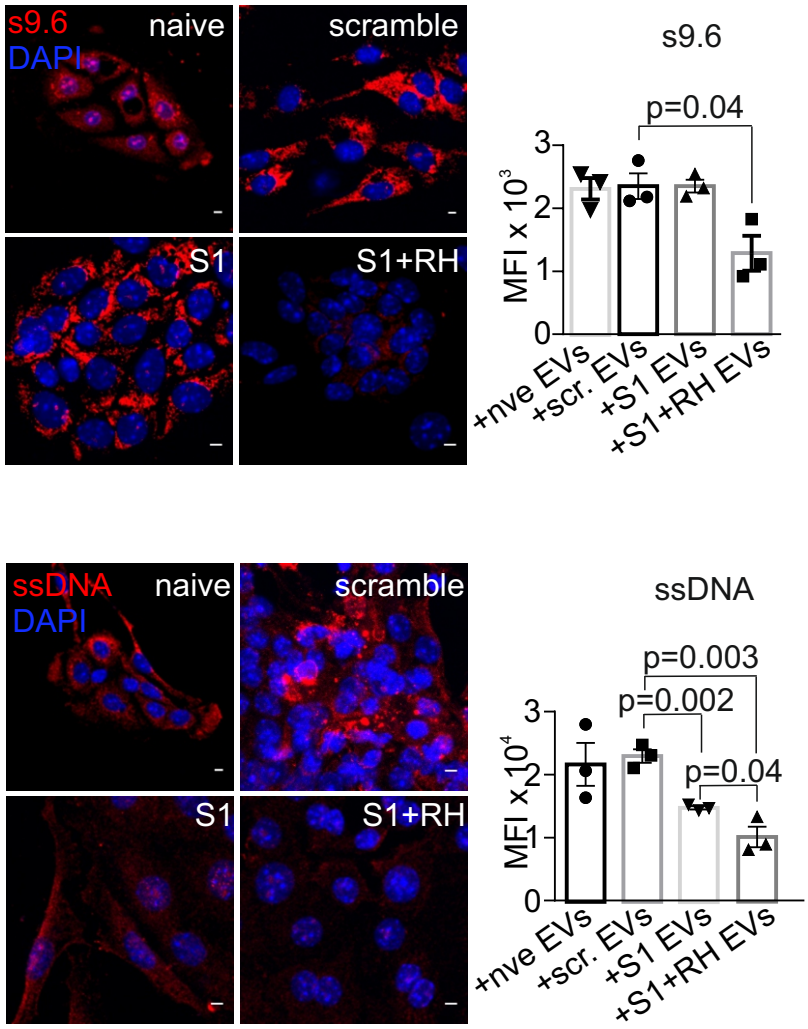


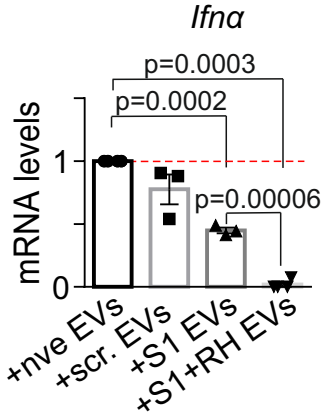
Supplementary Figure S5

Fig. S5.

Supplementary Fig. 5. (A). Immunofluorescence detection of ssDNA in wt and *Ercc1*$^{-/-}$ (*Er1*$^{-/-}$) PPCs cultured under serum-starved conditions (1% FBS, 18h). The graph depicts the mean fluorescence intensity (MFI) per cell (n=3). **(B)**. Immunofluorescence detection of ssDNA in wt and *Er1*$^{-/-}$ PPCs cultured with hydroxyurea (HU, n=3). The graph depicts the fold change mean fluorescence intensity (MFI) per cell of treated wt and *Er1*$^{-/-}$ PPCs. **(C)**. qPCR detection of ssDNA fragments of tRA-responsive (*Rarb*, *Stra6*) and non-responsive (*β-actin*) genes in untreated and PPCs treated with tRA and Illudin S for 16h, in the absence or presence of S1 nuclease. ssDNA template was normalized by cytoplasmic protein quantity. The red dashed line represents the untreated levels. **(D)**. LINE1 ORF1, ORF2, 5' UTR and 3' UTR RNA levels in wt untreated and PPCs treated with tRA and Illudin S for 16h, or wt and *Er1*$^{-/-}$ PPCs. The red dashed line represents the wt levels. **(E)**. Western blot of flow-throughs from S9.6 immunoprecipitation (DRIP) for XPG, XPF, RNase H1 (RNH1) and DNA Topoisomerase 1 (TOP1) in wt and *Ercc1*$^{-/-}$ pancreatic nuclear extracts with and without RNase H (RH) treatment (n=6). **(F)**. Immunofluorescence detection of ssDNA in *Er1*$^{-/-}$, untreated, MMC- (10μg/ml, 4h) and Illudin S-treated (30ng/ml, 3h) wt PPCs, cultured with or without the nuclear export inhibitor leptomycin B (LMB, 40nM). The graph depicts the mean fluorescence intensity (MFI) per cell nucleus (n=3). **(G)**. Immunoprecipitation (IP) using anti-ssDNA in cytoplasmic extracts from wt untreated or MMC-treated (10ng/ml, 4h) pancreata analyzed by western blotting for RPA70. The input (INP) and flow-through (FTH) are 1/20 of the extract used (n=3). Unless otherwise stated, all tissues and cells used are derived from P15 mice. White line is set at 5μm scale. Error bars indicate S.E.M. among n≥3 replicates. The significance is set at p-value ≤0.05 and where appropriate the exact p-value is shown (two-tailed Student's t-test).







f.

p values	gen.boundRPA70	cytoRPA70	ssDNA	S9.6	γH2AX-53BP1
untr vs tRA/illS 8h	0.687762335	0.001461326	0.010351	0.0632	0.0023608
untr vs tRA/illS 16h	0.031951127	0.002247266	0.004953	0.022733	0.000689412
untr vs tRA/illS 48h	0.017248107	0.000993979	0.003481	0.001047	0.005517344
ATRi vs ATRi+tRA/illS 8h	0.754872242	0.046097726	0.01078	0.052053	0.003703114
ATRi vs ATRi+tRA/illS 16h	0.056106725	0.640812671	0.003167	0.000377	5.28266E-05
ATRi vs ATRi+tRA/illS 48h	0.182376841	0.98114495	0.001156	0.003108	0.000443317
untr vs ATRi	0.081285637	0.004245156	0.319566	0.523203	0.996169823
tRA/ills 8h vs ATRi+tRA/ills 8h	0.033568733	0.288979942	0.733164	0.262451	0.041310789
tRA/ills 16h vs ATRi+tRA/ills 16h	0.035346266	0.006681123	0.056251	0.539765	0.00951307
tRA/ills 48h vs ATRi+tRA/ills 48h	0.020501541	0.008228823	0.026194	0.647363	0.009140917

Supplementary Figure S6

Fig. S6.

Supplementary Fig. 6. Immunofluorescence detection of **(A)**. genome-bound RPA70, **(B)**. cytoplasmic RPA70, **(C)**. cytoplasmic ssDNA, **(D)**. RNA:DNA hybrids (S9.6 antibody) and **(E)**. $\gamma H2AX^{+};53BP1^{+}$ foci in wt untreated and PPCs treated with tRA and Illudin S for 8, 16 and 48h, in the presence or absence of ATR inhibitor (ATRi, +1h pre-treatment). The graphs depict the mean fluorescence intensity (MFI) or foci per cell, as indicated (n=3). P values, depicted as asterisks (grey=between -ATRi conditions, black=between +ATRi conditions, blue=between -/+ATRi, same condition) in (A) – (E) are shown in **(F)**. **(G)**. Immunofluorescence detection of S9.6 antibody in PPCs from *Er1*$^{-/-}$ mice injected with not loaded (naive), or EVs loaded with denatured scramble protein (scr. EVs), recombinant S1 nuclease (S1 EVs) or S1 nuclease and RNase H (S1+RH EVs). The graph depicts mean fluorescence intensity (MFI) per cell nucleus (n=3). **(H)**. Immunofluorescence detection of ssDNA in PPCs from *Er1*$^{-/-}$ mice injected with not loaded (naive, nve EVs), or EVs loaded with denatured scramble protein (scr. EVs), recombinant S1 nuclease (S1 EVs) or S1 nuclease and RNase H (S1+RH EVs). The graph depicts mean fluorescence intensity (MFI) per cell nucleus (n=3). **(I)**. *Ifnα* mRNA levels in pancreata from *Er1*$^{-/-}$ mice injected with not loaded (naive), or EVs loaded with denatured scramble protein (scr. EVs), recombinant S1 nuclease (S1 EVs) or S1 nuclease and RNase H (S1+RH EVs). The red dashed line represents the levels of *Er1*$^{-/-}$ mice injected with naive EVs (n≥3). Unless otherwise stated, all tissues and cells used are derived from P15 mice. White line is set at 5μm scale. Error bars indicate S.E.M. among n≥3 replicates. The significance is set at p-value ≤0.05 and where appropriate the exact p-value is shown (two-tailed Student's t-test).

Data S1. (separate file)

Differentially expressed genes in *Ercc1*$^{-/-}$ (*Er1*$^{-/-}$) compared to wt pancreata. FDR: False discovery rate; FC: Fold change